BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Na^+-H^+ exchange and intracellular pH regulation in colonocytes from the chick

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Received 27 September 1996; revised 18 December 1996; accepted 20 December 1996

Abstract

The involvement of Na^+-H^+ exchange in chicken colonocyte homeostasis was investigated. Colonocyte pH (pH_i) was measured with 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). The proton ionophore FCCP reduced basal pH_i, indicating that cytosolic $[H^+]$ is not at electrochemical equilibrium across the membrane. External Na^+ removal decreased pH_i and subsequent addition of Na^+ returns pH_i towards its control value. The rate of pH_i recovery from an acid load was Na^+-dependent (K_m for Na^+, 24 mM) and inhibited by EIPA (IC_{50}, 0.18 μM). The initial rate of Na^+-dependent cell alkalization increased as the pH_i decreased from 7.2 to 6.6 (Hill coefficient, 1.88). Radioisotope flux studies revealed that an outwardly directed proton gradient transiently stimulated Na^+ uptake into BBMV isolated from the chick colon. EIPA and amiloride inhibited pH gradient-driven Na^+ uptake (IC_{50} of 4 μM and 32 μM, respectively). The K_m for Na^+ of pH gradient-driven Na^+ uptake was 6.8 mM. The Hill coefficient of the relationship between the initial rate of pH-driven Na^+ uptake and the intravesicular pH was 0.70. It is concluded that a Na^+-H^+ exchanger is involved in pH_i homeostasis in chicken colonocytes and that these cells possess at least two types of Na^+-H^+ antiporters with different sensitivity to EIPA and different kinetic parameters.

Keywords: Brush border; Vesicle; Colon; Sodium ion-proton exchanger; Amiloride; 5-(N-ethyl-N-isopropyl)-Amiloride

1. Introduction

The mammalian colon epithelia plays a major role in electrolytes and water homeostasis [1]. The principal means for salt absorption appears to be a combination of Na^+-H^+ and Cl^-/HCO_3^- exchange across the proximal colon and a Na^+ channel-mediated Na^+ absorption in the distal colon [1–3]. In birds, where the uretral urine runs retrograde into the coprodeum and colon, the large intestine is exposed to fluids of osmolarities and ionic composition widely different from the plasma. Several studies have revealed that NaCl is absorbed across the large intestine of the domestic fowl [4–7], though the mechanism(s) underlying salt absorption has not been elucidated.

In this study we have investigated the presence of a Na^+-H^+ exchange activity in the apical membrane of chicken colonocytes and its role in pH_i homeostasis.

2. Materials and methods

2.1. Intracellular pH measurements

Hubbard chickens, 4- to 6-weeks old, were killed by decapitation. Colonocytes were isolated by

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PII S0005-2736(96)00264-7

hyaluronidase incubation as described in Ref. [8]. Cell viability was assessed by determining the fraction of the cell population able to exclude 0.2% Trypan blue and usually ranged from 60% to 75%.

Intracellular pH (pH_i) was measured fluorimetrically at 25°C with 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), as described in Ref. [9]. Fluorescence ratios (500/450) were correlated with pH_i at the end of each recording session by permeabilizing the cell membrane with 70 μM digitonin and constructed a calibration curve. As previously reported for chicken enterocytes [10], this procedure underestimates the actual pH_i values by 0.15 pH units and a correction of 0.15 pH units was applied to the results.

The ammonium chloride pulse [11] was used to acidify the cells. Initial H^+ efflux rates (J_H^+) in nmol per 15 s per milligram protein were calculated according to the formula:

$$J_H^+ = (dpH_i/dt)V\beta_t$$

where V is cell volume (3.7 μl/mg protein) as determined previously [12], dpH_i/dt is the rate of change of internal pH and β_t is the total intracellular buffering capacity (in mM/pH unit). Since the current study was carried out in nominally HCO_3^--free solutions, βCO_2 was assumed to be negligibly small and β_t was taken to equal β_i.

The standard solution contained in mM, 80 NaCl, 1 $CaCl_2$, 100 mannitol, 3 K_2HPO_4, 1 $MgCl_2$, 20 Hepes-Tris (pH, 7.4), 0.5 β-hydroxybutyrate, 10 fructose, 1 L-glutamine, and 1 mg/ml bovine serum albumin. NaCl was replaced isosmotically by choline chloride in the Na^+-free solutions.

2.2. Brush-border membrane vesicles preparation (BBMV)

Hubbard chickens, 4–6 weeks old, were decapitated. The colon was rapidly removed, cut open longitudinally, rinsed with ice-cold saline solution, wrapped in aluminum foil, frozen in liquid nitrogen and kept at −80°C until use. Brush-border membrane vesicles (BBMV) were isolated from the colonic mucose, by the method described by Harig et al. [13], using Mg^{2+} precipitation instead of Ca^{2+} precipitation as described in Ref. [14]. Briefly, the tissue was defrosted in a solution (14 ml/g) containing, in mM, 50 mannitol and 2 Hepes/Tris (pH 7.0); the mucose was scraped off with a glass slide, homogenized with a Ystral Politron at setting 4, for 90 s and filtered through a nylon gauze. $MgCl_2$, at final concentration of 10 mM, was added to the homogenate and stirred for 20 min. The suspension was centrifuged at 1000 $\times g$ for 10 min, the resultant supernatant was centrifuged at 39 000 $\times g$ for 20 min and the resultant pellet was suspended in appropriate loading buffers. The suspension was homogenized with 20 up–down strokes with a glass Teflon Dounce homogenizer and centrifuged at 39 000 $\times g$ for 30 min. The pellet was resuspended in appropriate loading buffers. Unless otherwise stated the loading buffer consisted of, in mM, 200 mannitol, 20 potassium gluconate and 50 Mes/Tris (pH 5.5). The isolated apical membranes were made homogenous by passing them through a 25-gauge and a 26-gauge needle several times, and stored in liquid nitrogen until use. All the steps were carried out at 4°C.

2.3. Protein and enzyme assays

Protein was measured by the method of Bradford [15], using gamma globulin as the standard. Enzymatic activities were determined at 37°C. Citrate synthase activity was assayed by the method of Srere [16]. Na^+-K^+-ATPase and K^+-ATPase activities were assayed by the method of Del Castillo et al. [17] except that the assay buffer contained (in mM): 5 MgCl, 0.5 EDTA, 2 Tris/ATP, 50 Tris-HCl (pH = 7.2), with or without either 20 KCl or 20 KCl plus 100 NaCl. Na^+-K^+-ATPase activity was defined as the difference in activity measured in the presence of Na^+ plus K^+ plus Mg^{2+} and that measured in the presence of K^+ plus Mg^{2+}. K^+-ATPase activity was defined as the difference in activity measured in the presence of K^+ plus Mg^{2+} and that measured in the presence of Mg^{2+}. Sucrase activity was assayed by the method of Dahlquist [18]. K^+-ATPase and sucrase activities were also measured in the presence and absence of sodium dodecyl sulfate (SDS) at SDS/protein ratio of 0.75.

2.4. Uptake studies

Substrate uptake was measured at 25°C by a rapid filtration technique as described by Cano et al. [19].

The uptake was initiated by adding 10 μl of membrane vesicle suspension to 90 μl of uptake buffer.

Except where indicated otherwise, the uptake buffer consisted, in mM, 200 mannitol, 20 potassium gluconate, 0.045 valinomycin, 0.1 sodium gluconate, tracers of $^{22}Na^+$ and either 50 Mes-Tris (pH 5.5) or 50 Hepes-Tris (pH 7.4). The stop solution contained, in mM, 200 mannitol, 20 potassium gluconate and 50 Mes-Tris (pH 5.5). The amount of protein in the assay tube ranged from 100 to 150 μg/100 μl of uptake buffer.

2.5. Chemicals

$^{22}Na^+$ (carrier free) was purchased from Amersham, BCECF-AM from Molecular Probes (Eugene, OR), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and amiloride from Merck, Sharp and Dohme. FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone), valinomycin, digitonin, hyaluronidase and all the salts used in the current study were obtained from Sigma Chemical Co., Madrid, Spain. None of the chemicals used in the current work interfered with the BCECF fluorescence.

The BCECF-AM (1.45 mM), digitonin (14 mM) and valinomycin (3.6 mM) were prepared in DMSO. They were stored for up to 30 days at -20°C without loss in potency. Solvent concentration did not exceed 0.5% (v:v) and did not affect the fluorescence of BCECF.

2.6. Calculations and statistics

Results are expressed as mean $\pm$ S.E.M. The kinetic parameters (K_m, V_{max} and IC_{50}) were determined using non-linear regression program to best-fit the data (ENZFITTER program). Statistical significance was evaluated by the two-tailed Student's *t*-test for unpaired observations. In the figures vertical bars, that represent the S.E.M., are absent when they are less than symbol height.

3. Results

3.1. Intracellular steady-state pH and intracellular buffering capacity of colonocytes

The current work was conducted in the nominal absence of CO_2/HCO_3^- to minimize the contribution of HCO_3^--dependent transport mechanisms. Steady-state pH_i in colonocytes measured at 25°C, was 7.16 ± 0.02 ($n = 15$). Addition of FCCP rapidly decreased pH_i to a new steady-state pH_i value of 7.02 ± 0.03, $n = 4$ (Fig. 1A). However, addition of FCCP to acid-loaded cells (see below) results in cell alkalinization (see Fig. 2D).

Intracellular buffering power, β_i, was calculated from the value of pH_i reached following the addition of 20 mM NH_4Cl in cells exposed to an NH_4^+ pulse (0–40 mM of NH_4Cl to obtain different pH_i), both in the presence and absence of 50 μM EIPA and 0.1 mM ouabain. It was found that β_i increased (range: 93–109 mM/pH unit) as the pH_i decreased (range: 7.2–6.4). The mean value for β_i was 83 ± 7 mM/pH unit ($n = 18$). The inverse linear relationship between β_i and pH_i is given by the formula:

$$\beta_i = -20\ pH_i + 237 (r = 0.36)$$

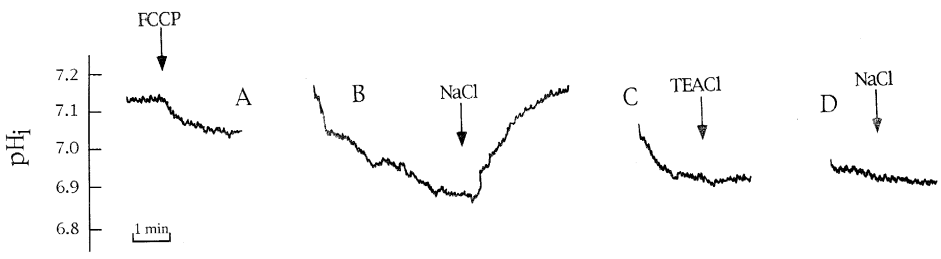

Fig. 1. Effect of FCCP and Na^+ removal on resting pH_i in isolated chicken colonocytes. A: dye-loaded cells incubated in standard solution were suspended in standard solution. B and C: at the beginning of each trace, dye-loaded cells incubated in standard solution were suspended in Na^+-free solutions (choline substitution). In D, cells were incubated for 5 min in standard solution containing 50 μM EIPA and suspended in Na^+-free solutions containing 50 μM EIPA. At the times indicated by the arrows 10 μM FCCP, 80 mM NaCl or 80 mM TEACl were added to the cuvettes. Each trace is representative of 5 independent experiments.

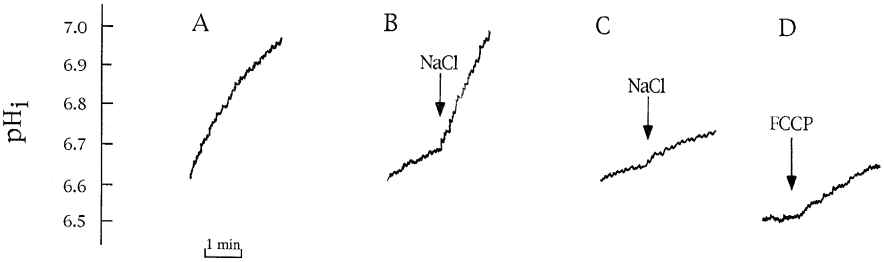

Fig. 2. External Na^+ and pH_i recovery from acid load. Dye-loaded cells were acidified by incubation at 25°C in NaCl-free solutions (choline substitution) containing 30 mM NH_4Cl for 10 min. Cells were then centrifuged and washed quickly in NH_4^+- and Na^+-free buffer (choline substitution), before being suspended in the appropriate solution. At the beginning of the trace cells were suspended in NaCl-medium (A) or in Na^+-free medium (B, C and D). In C, 50 μM EIPA was present during the incubation period and throughout the entire experiment. At the time indicated by the arrow 80 mM NaCl or 10 μM FCCP were added to the cuvettes. Each trace is representative of six independent experiments.

3.2. Na^+-H^+ exchanger and pH_i regulation

To test whether the chicken colonocytes possess a functional Na^+-H^+ antiporter at resting pH_i the effects of Na^+ removal on pH_i were investigated. Cells incubated in Na^+-containing solution were suspended in Na^+-free medium (choline substitution) at the beginning of the fluorescence recording. This maneuver would reverse the ion gradient for Na^+ and an operational Na^+-H^+ exchanger in the membrane would produce a net influx of H^+. When extracellular Na^+ was removed pH_i fell by approx. 0.25 units to a new pH_i value of 6.92 ± 0.02 (Fig. 1B). Readdition of Na^+ resulted in pH_i recovery. TEACl did not elicit pH_i recovery (Fig. 1C), suggesting that the effect of Na^+ was not due to changes in osmolarity or ionic strength of the suspending medium, but to a reversal of the transmembrane Na^+ gradient. Incubation with EIPA in Na^+-containing solution acidified the cells and Na^+-free conditions did not produce further acidification (Fig. 1D). Under these experimental conditions the readdition of Na^+ did not result in pH_i recovery.

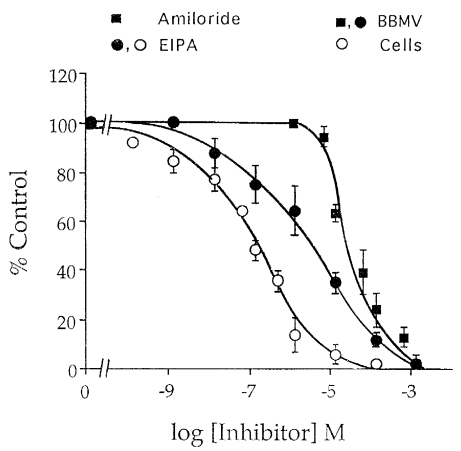

Fig. 3. Effect of EIPA and amiloride on either Na^+-dependent changes of pH_i after an acid load or on Na^+ uptake by colonic BBMV. In the fluorimetric studies, cells were acidified as explained in Fig. 2 to a pH_i value of 6.65 ± 0.01 ($n = 29$) and the rate of pH_i recovery was measured following the addition of NaCl (40 mmol/l). EIPA, at the desired concentration, was present during the NH_4Cl prepulse and throughout the entire experiment. Uptake of $^{22}Na^+$ was measured, in the presence and absence of a pH gradient, with increasing concentrations of either amiloride or EIPA. The membrane vesicles were preincubated for 5 min with the inhibitor. The results show the difference in Na^+ uptake measured in the presence and absence of pH gradient vs. inhibitor concentration. Means $\pm$ S.E. ($n = 6$) of rates of Na^+-induced pH_i recovery vs. inhibitor concentration.

The role of Na^+-H^+ antiporter in pH_i recovery

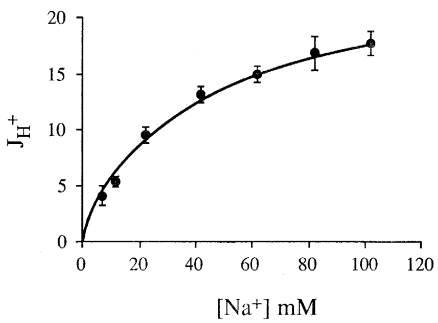

Fig. 4. Na^+-dependent changes of pH_i after an acid load. Cells were acidified as explained in Fig. 2 to a pH_i value of 6.90 ± 0.02. Means $\pm$ S.E. ($n = 5$) of rates of pH_i recovery vs. Na^+ concentration.

from an acid load was investigated in acidified colonocytes. Acid-loaded cells suspended in Na^+-containing solutions recovered towards the basal pH_i value with an initial rate of 0.055 ± 0.006 ($n = 10$) pH unit/15 s (Fig. 2A). Regulatory cell alkalization was inhibited, but it was not abolished, by either EIPA (Fig. 2C) or Na^+-free conditions (Fig. 2B). In the presence of 50 μM EIPA the initial pH_i recovery rate was 0.015 ± 0.003 ($n = 4$) pH unit/15 s (Fig. 2C).

The log–dose response curve of EIPA inhibition of the rate of Na^+-dependent pH_i recovery from an acid load, is shown in Fig. 3. The apparent half maximal inhibition (IC_{50}) for EIPA was 0.18 μM.

3.3. Kinetics of Na^+-H^+ antiporter involved in pH_i regulation

Acidified cells were added to an isosmotic Na^+-containing medium (various amounts of NaCl isosmotically replacing cholineCl). External Na^+ initiated concentration-dependent pH_i recovery (Fig. 4). The apparent K_m and V_{max} for Na^+ are given in Table 2.

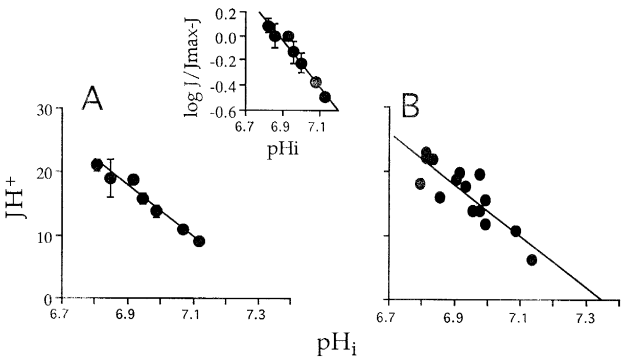

Fig. 5. Relationship between initial rate of Na^+-dependent pH_i recovery from an acid load and initial pH_i. Dye-loaded cells were acidified as described in Fig. 2 except NH_4Cl concentration prepulse was 15, 20, 25, 30 or 40 mM and incubation time ranged from 5 to 10 min to obtain different pH_i values. Acidified cells were added to cuvettes containing 80 mM NaCl. A: are means $\pm$ S.E.M. ($n = 5$) of rates of H^+ efflux (nmol/mg/15 s) against the means of pH_i at 0.05 pH unity intervals. B: All the individual data are plotted and adjusted to a line by computer. J_{max} was calculated from the Lineweaver-Burk plot of the data. Insert: Hill plot of data in A.

Table 1
Specific activities and enrichment factors of marker enzymes

Enzyme marker	Homogenate	BBMV	Enrichment factor
Citrate synthase	96 ± 3	0.72 ± 0.2	0.01
K^+-ATPase	4 ± 1	90 ± 1	22 ± 1
Na^+-K^+-ATPase	27 ± 2	41 ± 10	1.5 ± 1

Specific activities are expressed in nmol substrate $\cdot$ min^{-1} $\cdot$ mg of $protein^{-1}$. Enrichment factors were calculated by reference to the homogenate activity. Values are means $\pm$ S.E., $n = 5$.

3.4. Relationship between pH_i and Na^+-H^+ exchanger activity

The dependence of Na^+-H^+ exchange activity on pH_i was studied in colonocytes acidified by treatment with different concentrations of NH_4Cl. The relationship between net H^+ efflux ($nmol \cdot mg^{-1} \cdot 15\ s^{-1}$) and pH_i is shown in Fig. 5A. The Hill plot of the data (Fig. 5, inset) led to calculation of the Hill coefficient and the $[H^+]_{0.5}$ values (see Table 2). A Hill coefficient value greater than 1 indicates a positive cooperative mechanism for the influence of cytosolic protons on Na^+-H^+ antiporter. In Fig. 5B all the individual values are plotted and fitted to a line by computer. The extrapolated pH_i value at which no measurable cell alkalization occurred, set-point, is approx. 7.35.

3.5. Characterization of the preparation of brush-border membrane vesicles

By using a cytochemical method we have found that the apical membrane, but not the basolateral membrane, of chicken colonic epithelial cells presents a Na^+-independent, ouabain-sensitive, K^+,ATPase activity [20], and the measurement of the K^+,ATPase activity has been used to evaluate the purity of the colonic BBMV preparation.

The purity of the membrane vesicle preparations was evaluated by measuring the activity of markers characteristic of apical membranes (K^+-ATPase), basolateral membranes (Na^+,K^+,ATPase) and mitochondria (citrate synthase). The results are summarized in Table 1. The purification of brush-border membranes is consistent with the 22-fold enrichment of K^+-ATPase, the 1.5-fold enrichment of Na^+,K^+,ATPase and the lack of enrichment of citrate synthase. These results agree with those reported by

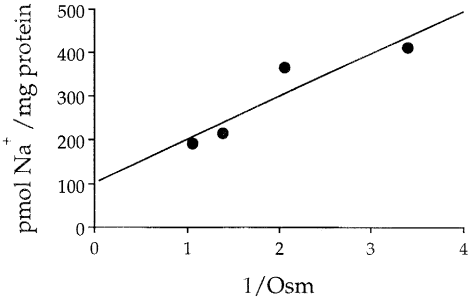

Fig. 6. Effect of external osmolarity on sodium uptake into BBMV isolated from chicken colonocytes. Medium osmolarity was increased by addition of mannitol. The composition of the intra- and extravesicular buffers are given in Section 2. Uptake of 0.1 mM Na^+ was measured during 1 h. Line was calculated by linear regression analysis. Each point represents the mean value ± S.E.M. of triplicate assays using three different membrane vesicle preparations.

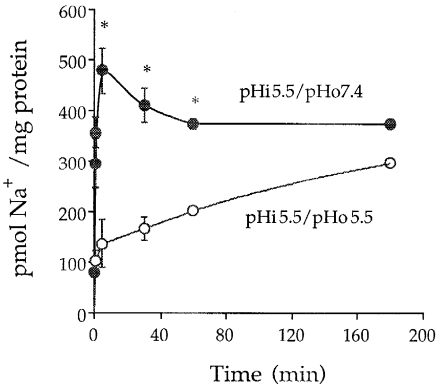

Fig. 7. Time course of Na^+ uptake by colonic BBMV. Na^+ uptake was measured in the presence (●) and absence (○) of a proton gradient. Other details are given in Section 2. Each point represents the mean value ± S.E.M. of triplicate assays using six separate membrane vesicle preparations. $^*P < 0.001$ as compared with the values obtained in the absence of pH gradient.

Del Castillo et al. [17] for rat distal colon. Sucrase activity was quite low (0.3 μmol/mg/min) and it was slightly increased (5% increase) by the presence of SDS in the assay buffer. The K^+-ATPase activity in nonpermeabilized membrane was ~25% of that measured in the presence of SDS. These results suggest that most (approx. 95%) of the vesicles were right-side out. The reason for the observed differences regarding the effect of SDS on enzyme activities could be that some of the vesicles are already open before adding the SDS.

3.6. Binding vs. transport of Na^+ into colonic BBMV

Nonspecific binding of $^{22}Na^+$ to the vesicle surface was evaluated by modifying the extravesicular osmolarity. Na^+ uptake at equilibrium (1 h) was decreased with increasing medium osmolarity (Fig. 6), indicating that Na^+ was taken into a closed intravesicular space. The relationship between Na^+ uptake and the reciprocal of osmolarity was linear and extrapolation of the line to infinite osmolarity (where intravesicular volume is zero) reveals that binding represents 24% of uptake at 340 mosmol/l. Binding was not affected by the presence or absence of a transmembrane pH gradient.

3.7. Na^+ uptake into BBMV isolated from the colon

A pH gradient (pH 5.5 inside, 7.4 outside) stimulated sodium uptake into the vesicles (Fig. 7). In the presence of a pH gradient sodium uptake overshoots the final steady-state equilibrium uptake value. No overshoot was observed in the absence of a pH gradient. The experiments were carried out with the voltage across the membranes brought to zero, by equal internal and external K^+ concentrations in the presence of valinomycin, to prevent the accumulation of negative charges inside the vesicles as the pH gradient dissipates. Electrodiffusional coupling was thus excluded as the cause for the pH gradient-driven sodium uptake.

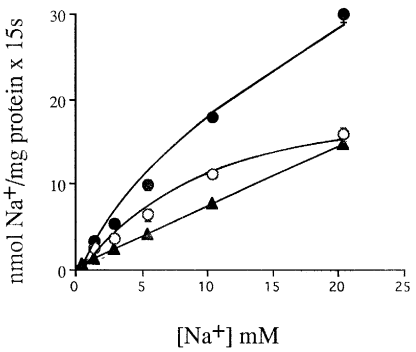

Fig. 8. Effect of increasing concentrations of external Na^+ on the initial rate of Na^+ uptake by colonic BBMV. Uptake buffer contained increasing concentrations of Nagluconate isosmotically substituted with mannitol. Uptake in the presence (●) and absence (△) of pH gradient. (○), difference: total uptake minus that in the absence of a pH gradient. Each point represents the mean value ± S.E.M. of triplicate assays using five independent membrane vesicle preparations.

Table 2
Kinetic parameters of the Na^+-H^+ exchange measured in chicken colonocytes and in the BBMV isolated from the chicken colon

	n	V_{max}	K_m	*n*	Hill coef.	$[H^+]_{0.5}$	*n*	EIPA IC_{50}
Colonocytes	5	0.02 ± 1	23 ± 3	5	1.88 ± 0.02	150 ± 10	5	0.18
BBMV	5	0.41 ± 0.05	6.8 ± 1.1	5	0.70 ± 0.10	302 ± 50 *	6	4

The apparent K_m (in mM), $[H^+]_{0.5}$ (in nM), V_{max} (nmol/mg/15 s), the IC_{50} (μM) and the Hill coefficient (coef.) values were calculated using non-linear regression analysis. *n*, is the number of independent experiments. The V_{max} obtained BBMV is referred to the protein in the initial homogenate, calculated from the protein recovery value. * $P < 0.05$.

pH-driven, membrane voltage-independent Na^+ uptake was inhibited by both, amiloride and EIPA, with an apparent half maximal inhibition (IC_{50}) value of 32 μM and 4 μM, respectively (Fig. 3).

3.8. Kinetics of the apical Na^+-H^+ exchanger of chicken colonocytes

Na^+-uptake into BBMV was measured at different external Na^+ concentrations in the presence and absence of an outwardly directed transmembrane proton gradient. In the presence of pH gradient, Na^+ uptake shows an inflexion at low Na^+ concentrations and it becomes a straight line at higher concentrations, not yielding a saturation curve (Fig. 8). In the absence of pH gradient, Na^+ uptake showed a linear relationship with its extravesicular concentration. The difference between total Na^+ uptake and that observed in the absence of pH gradient follows first-order kinetics. The apparent K_m and V_{max} for Na^+ are given in Table 2.

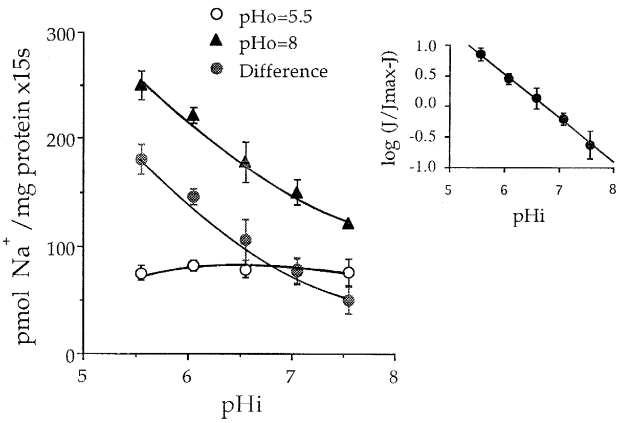

Fig. 9. Effect of intravesicular pH on the initial rate of Na^+ uptake. Vesicles were loaded with 200 mM mannitol, 20 mM potassium gluconate and 50 mM of either Hepes or Mes buffered with Tris to the indicated intravesicular pH. Uptake of 0.1 mM Na^+ was assayed in the presence of 200 mM mannitol, 20 mM potassium gluconate, tracers of $^{22}Na^+$ and either 50 mM Mes-Tris (pH_o 5.5, ○) or 50 mM Hepes-Tris (pH_o 8, △). Each point represents the mean value ± S.E.M. of triplicate assays using four independent membrane vesicle preparations. (●), Influx at pH_o 5.5 was subtracted from influx at pH_o 8 and the Hill plot of data is shown in the inset.

3.9. Na^+-H^+ activity and intravesicular pH

The sensitivity of the apical Na^+-H^+ exchanger of chicken colonocytes to pH was investigated by measuring Na^+ uptake into BBMV with the intravesicular pH preset to various values, and at two different external pH (pH_o) 5.5 and 8. At pH_o 8 Na^+ uptake was stimulated by decreasing internal pH (Fig. 9). The increase was completely inhibited at pH_o 5.5. The Hill coefficient and the $[H^+]_{0.5}$ values of the relationship between pH-driven Na^+ uptake and intravesicular pH are given in Table 2.

4. Discussion

Steady-state pH_i in chicken colonocytes was 7.16 ± 0.02 in the nominal absence of bicarbonate and at 25°C. This value agrees with available data of pH_i of most animal cells [21] including mammalian colonocytes [22,23]. In resting cells addition of FCCP rapidly decreased steady-state pH_i, whereas FCCP alkalinized acid-loaded cells. This indicates that FCCP is increasing the electrodiffusive H^+ flux, ruling out mitochondrial uncoupling, and suggesting that under resting conditions the transmembrane proton distribution is not at electrochemical equilibrium.

The observations listed below are consistent with a Na^+-H^+ exchanger involved in setting the resting pH_i and in pH_i recovery from an acid load: (1) intracellular acidification following Na^+-removal, (2)

return to resting pH_i values upon reinstatement of Na^+, but not upon addition of TEA, (3) inhibition of the Na^+-dependent pH_i changes by EIPA, with an apparent IC_{50} value of 0.18 μM, and (4) full recovery from acid loads required external Na^+ and it was inhibited by EIPA.

The following observations are consistent with the presence of a tightly coupled electroneutral Na^+-H^+ exchanger at the brush-border membrane of chicken colonocytes: (1) Na^+ uptake was stimulated by an outwardly directed proton gradient, (2) pH gradient-induced stimulation of Na^+ uptake was not due to generation of membrane potential, because it was observed under conditions of membrane depolarization, (3) pH-driven, membrane voltage-independent Na^+ uptake was inhibited by amiloride and EIPA. The IC_{50} values for both, amiloride or EIPA inhibition (32 μM and 4 μM, respectively) are comparable to those reported for the Na^+-H^+ exchanger located at the apical membrane of other epithelial cell types [24–26], including chicken enterocytes [27,28] and much higher than the values reported for the basolateral Na^+-H^+ isoform [24–26].

In epithelial cells Na^+-H^+ exchange activity has been found in both, the apical and basolateral membranes [24–26,29–33]. Several studies have shown that the apical and basolateral Na^+-H^+ exchanger are two distinct forms with different kinetic, biochemical and pharmacological characteristics, different intracellular signaling control and different cellular functions. The basolateral exchanger may represent a 'house-keeping' antiporter used for cell pH regulation and the apical may be involved in the transepithelial transport of salt and fluid.

Comparison of the kinetic parameters indicates that chicken colonocytes may have at least two types of Na^+-H^+ antiporters. As shown in Table 2 the Na^+-H^+ antiporter involved in pH_i homeostasis, but not that located on the apical membrane, of chicken colonocytes is allosterically regulated by intracellular pH (the Hill coefficient values were 1.88 and 0.70, respectively). These observations agree with previous reports showing that the apical Na^+-H^+ antiporter of rat colon [30] and of chicken enterocytes [27,28] are not modulated by pH_i. The apical exchanger is less sensitive to pH_i ($pK = 6.55 \pm 0.10$) than the exchanger involved in pH_i regulation ($pK = 6.82 \pm 0.03$). These two affinity constants closely agree with corresponding values reported for the rat NHE-3 ($pK = 6.45$) and NHE-1 ($pK = 6.75$) isoforms transfected in Na^+-H^+ antiporter-deficient Chinese hamster ovary cells [34]. The IC_{50} value for EIPA on the apical Na^+-H^+ antiporter was 4 μM and that for Na^+-induced pH_i recovery from an acid load was 0.18 μM. These values are of the same order of magnitude to those reported by Knickelbein et al. [25] for the apical and basolateral Na^+-H^+ antiporters of rabbit enterocytes. Finally, the K_m for Na^+ of the apical exchanger of chicken colonocytes is lower than that found for the antiporter involved in pH_i regulation. This agrees with previous reports showing that in rabbit ileum [25] and rat colon [29–31] the K_m for Na^+ of the apical Na^+-H^+ antiporter is lower than that of the basolateral antiporter. Also the K_m for Na^+ of the rat NHE-1 isoform is higher than that of the NHE-3 [34]. However, intact cells, unlike BBMV's, reflect transport processes occurring at both the apical and basolateral membranes. Therefore the K_m for Na^+ and IC_{50} for EIPA values obtained in intact cells are not purely those of the Na^+-H^+ antiporter involved in pH_i regulation, but those of the combined action of the apical and basolateral Na-dependent, EIPA-sensitive acid extrusion.

In conclusion the studies using spectrofluorimetry reveal the presence in chicken colonocytes of a Na^+-H^+ antiporter involved in pH_i regulation and the isotope flux studies reveal the presence of an apical Na^+-H^+ antiporter. The higher affinity for intracellular protons of the exchanger involved in pH_i regulation is consistent with its physiological role. On

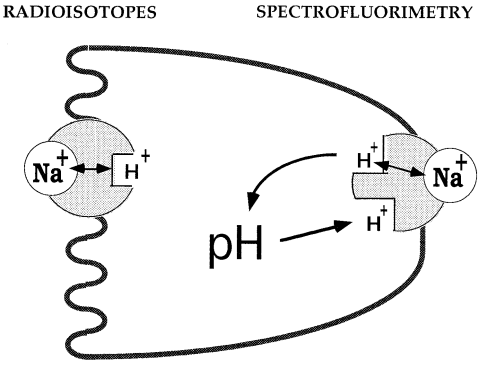

Fig. 10. Postulated Na^+-H^+ exchangers in chicken colonocytes. The apical Na^+-H^+ exchanger is not regulated by cytosolic pH, whereas this parameter allosterically regulated the basolateral exchanger.

the other hand, the higher affinity of the apical Na^+-H^+ antiporter for extracellular Na^+ suggests that the principal physiological role of this antiporter is the transepithelial Na^+ absorption, and hence, indicate that the Na^+-H^+ antiporter involved in pH_i homeostasis must be located at the basolateral membrane (Fig. 10). The current results, however, contrast with those recently reported by King et al. [23] showing that in rat colonocytes the Na^+-H^+ exchanger involved in pH_i homeostasis is present in the apical membrane but not in the basolateral membrane.

Acknowledgements

The work was supported by a grant from the Spanish DGICYT PB92-0690. We thank Dr. S.P. Shirazi-Beechey for kindly advising us to use Mg^{2+} precipitation instead of Ca^{2+} precipitation.

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